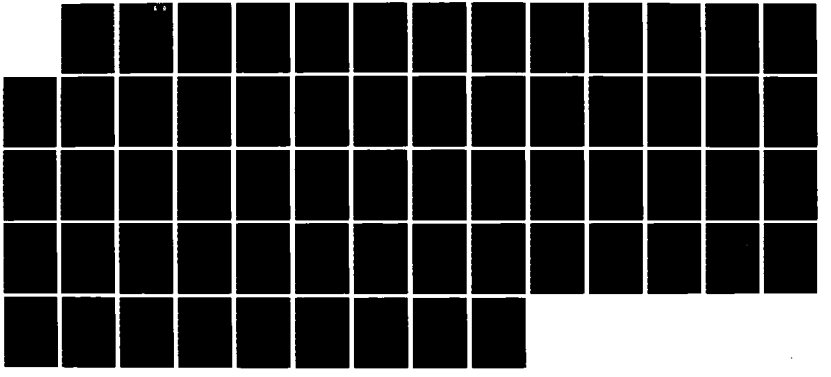
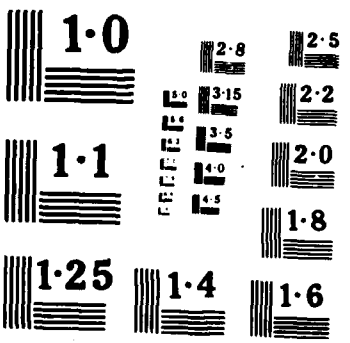
AD-A170 020 AN INVESTIGATION INTO THE EFFECTS OF PEPTIDE NEUROTRANSMITTERS AND INTRAC..(U) AT AND T BELL LABS INC MURRAY HILL NJ J A CONNOR 21 MAY 86 1/1

UNCLASSIFIED AFOSR-TR-86-0466 F49620-85-C-0009 F/G 6/3 NL

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## REPORT DOCUMENTATION PAGE

# AD-A170 020

1b. RESTRICTIVE MARKINGS

3. DISTRIBUTION/AVAILABILITY OF REPORT
Approved for public release; distribution unlimited

2b. DECLASSIFICATION/DOWNGRADING SCHEDULE

4. PERFORMING ORGANIZATION REPORT NUMBER(S)

5. MONITORING ORGANIZATION REPORT NUMBER(S)
AFOSR-TR- 86-0466

6a. NAME OF PERFORMING ORGANIZATION
AT&T Bell Labs

6b. OFFICE SYMBOL (If applicable)

7a. NAME OF MONITORING ORGANIZATION
Air Force Office of Scientific Research

6c. ADDRESS (City, State, and ZIP Code)
600 Mountain Avenue
Murray Hill, NJ 07974

7b. ADDRESS (City, State, and ZIP Code)
Building 410
Bolling AFB, DC 20332-6448

8a. NAME OF FUNDING/SPONSORING ORGANIZATION
AFOSR

8b. OFFICE SYMBOL (If applicable)
NL

9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
F49620-85-C-0009

8c. ADDRESS (City, State, and ZIP Code)
Building 410
Bolling AFB, Dc 20332

10. SOURCE OF FUNDING NUMBERS

| PROGRAM ELEMENT NO. | PROJECT NO. | TASK NO. | WORK UNIT ACCESSION NO. |
|---|---|---|---|
| 61102F | 2312 | K2 | |

11. TITLE (Include Security Classification) An investigation into the effects of peptide neurotransmitters and intracellular second messengers in rat central neurons in culture.

12. PERSONAL AUTHOR(S)
John A. Connor

| 13a. TYPE OF REPORT | 13b. TIME COVERED | 14. DATE OF REPORT (Year, Month, Day) | 15. PAGE COUNT |
|---|---|---|---|
| Annual | FROM 10/15/84 TO 10/15/85 | 5/21/86 | |

16. SUPPLEMENTARY NOTATION

| 17. COSATI CODES | | |
|---|---|---|
| FIELD | GROUP | SUB-GROUP |
| | | |
| | | |

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
intracellular calcium; second messengers; cerebellum; diencephalon; peptides; cAMP; GABA; glutamate; histamine presynaptic inhibition

19. ABSTRACT (Continue on reverse if necessary and identify by block number)
Significant progress has been made in several areas of our research proposal. We have developed a Ca-imaging system which is now capable of resolving subcellular Ca changes on the order of seconds. This Ca-imaging system has been used to study neurotransmitter actions in cultured diencephalic and cerebellar neurons. Both classical (GABA) and modulatory (thyroid hormone) neurotransmitters were found to affect cellular $Ca^{2+}$ levels in these cells without depolarizing resting potential. We have also continued studies of second messenger action in identifiable molluscan neurons. The concentration of endogenous cyclic AMP in single cells was measured, and under stimulated conditions the levels were sufficient to suggest activation of cyclic AMP-induced membrane conductances.

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20. DISTRIBUTION/AVAILABILITY OF ABSTRACT
☒ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS

21. ABSTRACT SECURITY CLASSIFICATION
Unclassified

| 22a. NAME OF RESPONSIBLE INDIVIDUAL | 22b. TELEPHONE (Include Area Code) | 22c. OFFICE SYMBOL |
|---|---|---|
| Dr. William O. Berry | (202) 767-5021 | NL |

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

ANNUAL TECHNICAL REPORT

F49620-85-C-0009 : AN INVESTIGATION INTO THE EFFECTS OF PEPTIDE NEUROTRANSMITTERS AND INTRACELLULAR SECOND MESSENGERS IN RAT CENTRAL NEURONS IN CULTURE.



## SUMMARY

Significant progress has been made in several areas of our research proposal. We have developed a Ca-imaging system which is now capable of resolving subcellular Ca changes on the order of seconds. This Ca-imaging system has been used to study neurotransmitter actions in cultured diencephalic and cerebellar neurons. Both classical (GABA) and modulatory (thyroid hormone) neurotransmitters were found to affect cellular $Ca^{2+}$ levels in these cells without depolarizing resting potential. We have also continued studies of second messenger action in identifiable molluscan neurons. The concentration of endogenous cyclic AMP in single cells was measured, and under stimulated conditions the levels were sufficient to suggest activation of cyclic AMP-induced membrane conductances.

## RESEARCH OBJECTIVES

The primary objective of this research is to elucidate how endogenous neuroactive substances (i.e., neurotransmitters, neuromodulators, second messengers) affect brain function. Nerve cells are the functional units of the brain, and changes in neuronal activity are ultimately expressed through modifications in membrane conductances. We have therefore focused our attention on examining the mechanisms by which neuroactive substances alter membrane conductances.

A central tenet of our research has been the utility of using specific types of nerve cells, ones which can be readily identified using morphological and electrophysiological criteria. We have continued studies using giant molluscan neurons which are relatively easy to identify, and we have initiated experiments aimed at identifying appropriate mammalian neurons for

our studies. In the mammalian CNS it is virtually impossible to analyze membrane conductance changes *in situ*. Instead our aim has been to combine cell culturing techniques with immunostaining of cells in order to study identifiable mammalian neurons *in vitro*.

The immediate goals of this research were therefore centered around identifying mammalian neurons *in vitro*. Several regions from the developing rat brain were selected which are known to have specific nerve cell types which respond to neuroactive agents. The regions and cell types selected were hippocampal pyramidal and granule cells, hypothalamic magnocellular neurons, and cerebellar granule and Purkinje cells. We have attempted to identify each of these cell types in culture using morphological and immunocytochemical criteria.

The long-term goals of this research are to analyze the effects of various neurotransmitters (classical and neuromodulatory) on the cellular properties of identified molluscan and mammalian neurons. Using advanced electrical recording and optical techniques, patch and voltage clamping, intracellular indicator dyes, voltage sensing dyes, and high resolution image processing, we will define the specific modifications brought about by these neurotransmitters on nerve excitability, synaptic interactions, and cellular morphology. By understanding the mechanisms of these modificiations one can begin to comprehend the wide diversity of effects that endogeneous neuroactive agents have on brain functions, including behavior.

## SPECIFIC OBJECTIVES

1. Develop cell culture techniques for isolation and characterization of mammalian CNS neurons
2. Develop immunocytochemical methods for identification of cell types
3. Assess cell viability under various culture conditions, including serum-free medium
4. Analyze growth characteristics of cells *in vitro*

5. Monitor cellular excitability *in vitro*

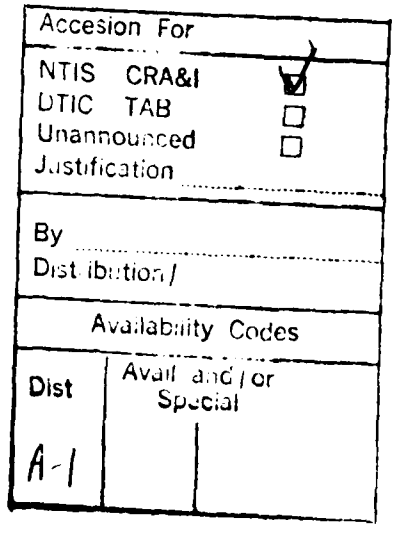
6. Characterize the types of membrane conductances expressed

7. Examine the general effects of neurotransmitters (GABA, glutamate, histamine, biogenic amines, peptides) on excitability and membrane conductances

8. Examine the effects of second messengers on excitability and membrane conductances

9. Monitor changes in intracellular $Ca^{2+}$ changes during spontaneous electrical activity or application of neurotransmitter agents

10. Analyze the role of neurotransmitters and $Ca^{2+}$ in synaptic plasticity (e.g. presynaptic inhibition)



## STATUS OF THE RESEARCH

During the first 12 months significant progress has been made toward realizing the goals of our research proposals. Studies on each of our ten specific objectives have been initiated, and results from several of these projects have been submitted for publication in scientific journals. Our most important accomplishments can be categorized into four areas: (1) further development of a Ca-imaging system now capable of resolving subcellular Ca changes on the order of seconds; (2) analysis of peptide function in cultured mammalian diencephalic neurons; (3) investigation of neurotransmitter actions in cultured cerebellar granule cells; and (4) elucidation of neurotransmitter and second messenger actions in identifiable molluscan neurons.

### A. Ca-Imaging of Growth Cones

A major effort of the laboratory has been the development of a Ca-imaging system for monitoring changes in cellular Ca levels. A high resolution digital imaging system is presently operational, and it employs the fluorescent Ca-indicator dyes, quin-2 and fura-2 (Grynkiewicz, Poenie and Tsien, *J. Biol. Chem.* 260: 3440-3450, 1985). Ken McMillan from Stanford University was hired for a week in June to write an expanded data acquisition and analysis program for the system.

We have used this imaging system to monitor spatially-resolved $Ca^{2+}$ changes in cultured cells with a time resolution of a few seconds (Connor, *Soc. Neurosci. Abstr.* 11: 176, 1985; Connor, *PNAS*, in press). The indicators can be easily loaded into cells whereupon it undergoes large, reversible fluorescent changes upon binding $Ca^{2+}$. We have been able to show conclusively that the growing tips of cell processes (i.e., growth cones) contain very high free Ca levels compared to other non-growing regions of the cell. Actual concentrations of free $Ca^{2+}$ have been estimated to be several hundred nanomolar in the growing tips and 50-100 nM elsewhere

in the cell.

B. Peptide Function in Cultural Diencephalic Cells

We have used immunocytochemical, electrophysiological, and optical measurements of dissociated diencephalic cells from embryonic rat brain (gestation day 18) grown in defined medium. The immunocytochemical studies have involved peroxidase - antiperoxidase (PAP) staining for neuropeptide production over time in culture. Hsiu-Yu Tseng (Bell Laboratories) has found that selective staining of some cells occurs after 11 days *in vitro* (DIV) using PAP-conjugated antibodies to ACTH, beta -endorphin, substance P, and leu-enkephalin. Negative results were obtained using antibodies to insulin, met-enkephalin, neurotensin, prolactin, and human growth hormone. ACTH was present in the magnocellular neurons which could be identified after 24 DIV. Experiments are in progress to identify the subregion of diencephalon which produces these magnocellular neurons (current suspicion is the hypothalamus).

This preparation has also been used to study the action of thyroid hormones, T3 and T4, on the electrical properties and intracellular Ca levels of the diencephalic cells. This project has been done in collaboration with Dr. D. Tank (Bell Laboratories) and Drs. Z. Ahmed and L. Kragie-Ahmed (SUNY-Buffalo). Using whole-cell patch recording we have characterized the development of voltage-dependent membrane conductances in these cells *in vitro* (Ahmed, Tank and Connor, Devel. Brain Res., in press). Analyses of the effect of T3 or T4 on these conductances have indicated no change in resting potential as well as no apparent change in the properties of Na-or K-conductances. The Ahmeds have found biochemical evidence indicating that these hormones increase active transport of Ca out of these cells via Ca-ATPase. We have performed preliminary experiments using the Ca-imaging system which indicate that calcium levels decrease upon exposure to T4 (Kragie-Ahmed, Ahmed, Connor and Davis, *Soc. Neurosci. Abstr.* 11: 543, 1985).

C. Neurotransmitter Actions in Cultured Cerebellar Cells

Using immunocytochemical and electrophysiological methods we have been able to identify and study cerebellar granule cells in culture. This cell type is the most numerous in the mammalian brain, equivalent in number to all other neurons combined in most mammals. In spite of its preponderance, there are currently no intracellular records from this cell type nor any evidence regarding the types of membrane conductances present. Phil Hockberger has developed an explant culture preparation using newborn rat cerebellum which allows apparently normal granule cell development to be studied *in vitro*. Using whole-cell patch recording he has made both intracellular recordings and voltage clamp analyses of the development of specific membrane conductances (Hockberger and Connor, *Soc. Neurosci. Abstr.* 11: 508, 1985).

Cultured granule cells were excited by iontophoretically-applied glutamate and inhibited by GABA. We have begun to examine the effects of these neurotransmitters on membrane conductance and intracellular $Ca^{2+}$ levels. Preliminary experiments using the Ca-imaging system indicate that both types of transmitters can elevate intracelluler $Ca^{2+}$ levels even though the GABA response occurs in the absence of a membrane potential change.

D. Analyses of Neurotransmitter Actions and Second Messenger Effects in Molluscan Neurons

This research is a continuation of on-going studies in our laboratory using giant molluscan neurons. Phil Hockberger and Tets Yamane (Bell Laboratories) have used single cell isolation and radioimmunoassay techniques to quantify changes in the concentration of cyclic AMP in single *Aplysia* cells exposed to neurotransmitters and other chemical agents (Hockberger and Yamane, submitted).

They have shown that resting levels of cyclic AMP (roughly 1-5 mu M) can be elevated several-fold by phosphodiesterase inhibitors at concentrations which effect neuronal

excitability. The cellular cyclic AMP concentrations were elevated under such conditions to values which overlap with levels obtained with cyclic AMP injections. The latter have previously been shown to induce changes in membrane sodium conductance in the same neurons (Connor and Hockberger *J. Physiol.* 354: 139-162, 1984). Their results support the idea that endogenous cyclic AMP can also induce changes in membrane sodium conductance in these cells.

We are also studyng the actions of histamine on intracellular calcium, and on a form of synaptic plasticity, presynaptic inhibition. The best system for studying the role of histamine as a neurotransmitter is in the nervous system of *Aplysia.* A neuron (C2) has been shown by several criteria to use histamine as its transmitter. In addition, histamine released by this neuron Reduced the amount of transmitter released by other identified neurons (i.e., it causes presynaptic inhibition). Since transmitter release requires an influx of calcium into the synaptic terminal, histamine must be acting, directly or indirectly, on intracellular calcium. Hillel Chiel has begun to study the role that histamine may play in regulating intracellular calcium in identified *Aplysia* neurons using the calcium-sensitive dye Arsenazo III. In collaboration with David Tank, he has also begun to culture *Aplysia* neurons which are involved in presynaptic inhibition, so that he can study the biophysical properties of this phenomenon more directly.

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1. Connor, J. A. (1985). $Ca^{2+}$ measurements using the fluorescent indicators quin 2 and fura 2 combined with digital imaging in mammalian CNS cells. *Soc. Neurosci. Abstr.* 11: 176.

2. Connor, J. A. (1986). Digital imaging of free calcium changes and of spatial gradients in growing processes in single, mammalian CNS cells. *PNAS* (in press).

3. Ahmed, Z., Tank, D. and Connor, J. A. (1986). Development of Membrane Currents in fetal rat neurons. *Develop Brain Res.* (in press).

4. Kragie-Ahmed, L. Ahmed, Z., Connor, J. A. Davis, P. J. (1985). Neuronal Ca ATPase activity and changes in intracelluelar $Ca^{2+}$ induced by short-term exposure to thyroid hormone. *Soc. Neurosci. Abstr.* 11: 543.

5. Hockberger, P. and Connor, J. A. (1985). Properties of cultured cerebellar granule cells measured with whole-cell patch recording and imaging of $Ca^{2+}$ indicator fluorescence: *Soc. Neurosci. Abstr.* 11: 508.

6. Hockberger, P. and Yamane, T. (1986). Estimation of total protein content and free cyclic AMP concentration in ganglia and single neurons of *Aplysia California.* (submitted to *Brain Research*)

## SEMINARS

1. University of Maryland Medical School, Dept. of Physiology, Dec. 18, 1985.

2. Cornell University, Dept. of Applied Physics, Dec. 4, 1985.

3. Columbia University, Center for Neurobiology & Behavior, Dec. 12, 1985.

4. Yale University, Division of Neurobiology, July 13, 1985.

## PERSONNEL

John A. Connor, Ph.D. (principal investigator)

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Hillel Chiel, Ph.D.

Hsiu-Yu Tseng, M.S. (Bell Laboratories)

Kenneth McMillan, B.S. (Stanford)

Zahur Ahmed, Ph.D. (SUNY-Buffalo)

PNAS (USA) — in press

# Digital Imaging of Free Calcium Changes and of Spatial Gradients in Growing Processes in Single, Mammalian CNS Cells

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Index words: Growth cones, digital imaging, $Ca^{2+}$, fluorescent indicators

# Digital imaging of free calcium changes and of spatial gradients in growing processes in single, mammalian CNS cells

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*ABSTRACT*

Intracellular free calcium levels have been measured in cultured CNS cells using the fluorescent indicator fura-2 and digital imaging techniques. Cells were plated from rat embryo diencephalon (E-17,18), nearly all the cells surviving dissociation having undergone final mitosis within the previous 24 hours. The initially spherical cells were observed within the first 24 hours in culture when they were extending processes, but had not established a network of fibers that would prevent the identification of the origin of a given fiber. Cells that were rapidly extending showed high $Ca^{2+}$ levels in the regions of growth. Where processes had just emerged from the soma or where growth was proceeding from more than one pole, $Ca^{2+}$ levels were uniform and estimated levels of 500 nM were commonly seen. In active growth cones distant from the soma, $Ca^{2+}$ levels exceeded 200 nM while the soma levels were in the 60-80 nM range. Non-extended and extended cells that had stalled had uniform $Ca^{2+}$ levels in the range of 30-70 nM. The results show that high $Ca^{2+}$ levels are at least a correlate of extension in CNS cells and that under some conditions the region of high calcium can be localized to a small part of the cell.

# Digital imaging of free calcium changes and of spatial gradients in growing processes in single, mammalian CNS cells

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An understanding of the dynamics of outgrowth in cells of the mammalian central nervous system (CNS) is a critical prerequisite to understanding cell recognition and pattern formation within the CNS. Among the factors that may control outgrowth in developing neurons and other cells is the internal free calcium level in extending regions (growth cones). Elevated calcium levels, by analogy to other motile and secretory systems, could allow the local activation of actinomyosin systems to stretch or shape a growing region, or promote the insertion of new membrane, as in vesicular secretion, and thereby produce outgrowth. Recent electrophysiological data indicate that transmembrane Ca transport activity exists in growth cones (4, 8, 10, 14, 18, 20) suggesting the possibility of elevated $Ca^{2+}$ concentration. The present study has employed fluorescent Ca indicators and a cooled, charge-coupled device (CCD) camera of the type used in astronomy over the past several years (26, 27) to image free $Ca^{2+}$ levels in individual cultured CNS cells from rat embryo. This combination has made it possible to follow short term changes in the spatial distribution of $Ca^{2+}$ during physiological events in single cells. It is shown that cells undergoing rapid extension display very high internal $Ca^{2+}$ levels, and that where growing processes have extended a significant distance from the soma, the high level is localized to the growing tips. Cells in which growth has been arrested show low internal $Ca^{2+}$ that is uniform throughout the cell.

The fluorescent Ca indicator quin2 (19, 24) has proved extremely useful in the measurement of calcium ion concentrations in populations of small cells during physiological activity. Fura-2, a recently developed and related indicator, is an even more powerful probe for following $Ca^{2+}$ changes in cells, owing chiefly to its brighter fluorescence and high selectivity for $Ca^{2+}$ over other ions (11, 25). As an acetoxymethyl ester (quin 2/AM or fura-2/AM) either dye freely crosses cell membranes. Once in the cytoplasm either is deesterified and is thereby trapped. This loading technique has greatly broadened the classes of cells in which internal free calcium ($Ca^{2+}$) can be monitored. More conventional indicators such as arsenazo III or aequorin, commonly used on single

cells, must be injected through microelectrodes, which has limited their use to cells with a feature greater than about 30 μm in diameter, or else must be introduced by chemically permeablizing cells, which may in some cases have unwanted side effects (12, 7, 21). The use of high resolution imaging technology opens the possibility of detecting cell to cell variations in free calcium as well as differences within cells. The available imaging data suggest that such differences do exist (5, 6, 15, 17, 28). These considerations are of considerable importance in neurobiology where it often seems necessary to deal with mixed populations, different glial and neural types, in order to maintain development and differentiation. In addition, much of nerve cell function (such as secretion or growth), linked to $Ca^{2+}$, is locally expressed, raising the possibility of considerable intracellular $Ca^{2+}$ concentration gradients.

*METHODS*

Cells from embryonic rat (E-17 or E-18) diencephalon were trypsin-dispersed, plated on #1 glass coverslips coated with poly-D-lysine at $2 \times 10^3$ cells/mm$^2$, and maintained in serum-free defined media as described elsewhere (2). Fura-2/AM (Molecular Probes, Junction City, OR) was dissolved in DMSO, 5 mg/ml to make a stock solution. Loading solutions were made by adding the stock to the serum-free defined medium to give nominal concentrations of 4 to 6 μM. Cells were bathed in the loading solution for 25 to 30 minutes at 36°C, then rinsed and given a 1.5-3 hr post-incubation in defined medium to allow deesterification of the indicator.

Plated cells were mounted in a temperature-controlled chamber (33-36°C) containing either Krebs saline or defined media that allowed direct access to the coverslip underside, and placed on the stage of a Zeiss IM-35 microscope. A Nikon UV-F 40X glycerine immersion objective was used for epifluorescence measurements together with a 100 watt Hg lamp (Osram) mounted in the Zeiss housing. The CCD camera (Photometrics Ltd, Tucson Ariz, model 81-A) employed an RCA SID 53612 chip, 320 x 512 pixels. Exposure times of 0.2 to 0.5s were employed depending upon the size of cell, dye loading, and contrast desired. Apparent bleaching of fura-2 was less than 5% for 20 s exposures to 340 nm excitation at the intensity used in experiments. Excitation wavelengths at 340, 360, and 380 nm were obtained using interference filters (Melles-Griot, 10 nm 1/2 B.W.).

Exposure times were controlled by a Uniblitz shutter mounted between the filters and the lamp condenser lens. A neutral density (1.5 OD) filter was used in conjunction with the 360 and 380 nm filters to obtain approximately equal fluorescence image intensity for the two excitation wavelengths. The emission spectrum of fura-2 is centered at 500 nm and after numerous studies employing an interference filter (500 nm, 50 nm bandwidth) in the light exit path, most experiments were conducted with a 480 nm long pass exit filter to maximize light transmission. Paired exposures were separated by approximately 1.5s, the time required to change excitation filters. Each frame was corrected for camera dark current and background fluorescence by subtracting a frame of the same excitation wavelength made using a blank coverslip covered to an appropriate depth with the same media that bathed the cells. Blanks were made each experimental day. Measurements have for the most part employed 340 and 380 nm excitation (nominal, see below), the maximally sensitive wavelengths on either side of the 360 nm crossover point. The formation of *ratio images* should, in principle, normalize for the effects of preparation geometry, scattering and illumination non-uniformity (see 11 and below).

In forming ratios of the data arrays, non-cellular areas present a problem in that the representations of light levels are small numbers and are therefore subject to large *relative* variation due to noise. Ratioing a field of these numbers gives an extremely mottled picture. For display purposes, a threshold is declared for the denominator field, and for pixel values below this, the ratio is replaced by a common, arbitrary value corresponding to a color or grey level. For color displays, black has been chosen (Figs. 1, 3) and an intermediate shade of gray in Fig. 2B.

For standardizations of fluorescence ratios and to set bounds on trapped dye concentration, glass coverslips (#1) with channels 2.7 μm ± .2 μm deep and 50 μm wide were prepared by chemical vapor etching. One of these coverslips clamped against a plain #1 coverslip (etched plate on top, channels inward) gave a cuvette with light path of 4 to 7 μm depending on how closely the unetched glass surfaces could be apposed. Actual depth for a given run was calculated from the known channel depth and the two fluorescence values of the filling solution at channel and non channel areas. Temperature was controlled by heating a pool of water on the top surface to 38°C.

Approximate cell thicknesses were determined by carefully focussing up and down with Nomarski optics at 400X magnification. Newly plated cells (< 24 hr in culture) were generally between 5 and 8 μm thick at the soma. A test media to which fura-2 and calcium buffers were added of the following composition was used: KCl, 110 mM; NaCl, 20 mM; $MgCl_2$, 1.2 mM MOPS buffer, 10 mM, pH = 7.10. Fluorescence of indicator-loaded cells were compared with that of the cuvette channels filled with fura-2 dissolved in the test media at known concentrations. These measurements used 360 nm excitation of fura-2 where fluorescence is independent of Ca and gave values in the range of 100 to 300 μm concentrations for newly plated cells. I have observed over the course of loading several different types of cells, and ages in culture, that the ultimate amount of dye trapped does not depend simply on the loading solution concentration and exposure time.

Calcium stocks were prepared using either EGTA or BAPTA (Molecular Probes) as calcium buffers. Indicator constants for the equation below (see 11) were determined on the microscope in 4 separate runs.

1) $$[Ca] = K_D \left( \frac{R - R_{min}}{R_{max} - R} \right) (F_0/F_s) ,$$

Minimum fluorescence ratio ($R_{min} = F_{340}/F_{380}$) were between 0.39 and 0.42 for test solutions with no added Ca and 5 mM EGTA or BAPTA. Maximum ratios ($R_{max}$) were between 9 and 10 for saturating levels of Ca. The ratio of fluorescence at 380 nm excitation at the limiting low and high Ca levels ($F_0/F_s$) was approximately 7.5. Intermediate ratios were measured using EGTA or BAPTA buffered test media. EGTA or BAPTA concentrations were between 5 and 8.3 mM with fura-2 concentration 150 uM, making it a major buffering component in the mix. Three sets of buffered Ca solutions were checked with an ion sensitive electrode (Dr. Simon Levy, Boston Univ.) to control for errors in making up the stocks. Conversion of ratios to $[Ca^{2+}]$ in the text are made using Eqn. 1 with $R_{min} = 0.4$, $R_{max} = 9.5$, $F_0/F_s = 7.5$ and $K_D = 225$. Electrode determinations of free Ca were within 0.1 pCa of levels calculated from equation 1 with the above constants over the range 6 to 7.5 pCa. The total dynamic range of the indicator, in cuvette, in the present instrument, $R_{max}/R_{min}$, is approximately 20, considerably less than the value measured in a standard

spectrofluorimeter (see also 11). This difference should arise from a shift of the nominal 340 and 380 bands toward the powerful 366 nm Hg line in the microscope apparatus.

In the diencephalon cells, cerebellar granule neurons (Hockberger & Connor in preparation), and adrenal glomerulosa cells (Connor, Cornwall & Williams, in preparation), the minimum fluorescence ratios ($R_{min}$) observed were 0.6-0.65. Maximum ratios, $R_{max}$, observed in response to elevated potassium or the calcium channel agonist, leptinotarsin, were 5 to 6. The problem of anomalously low ratio values in unstimulated cells as reported by Almers and Neher (ref. 3, methods) in mast cells was never encountered. At the upper end, the cuvette fluorescence ratios measured in 4:1 CaEGTA:EGTA buffer mixes (0.8-0.9 uM Ca, the highest buffered levels mixed) was 3.5-4. Ratios of 5 to 6 would predict Ca levels well above 1 uM and in many cases where leptinotarsin (16) was used to increase intracellular Ca, and the ratio tended beyond this limit, cell lysis was common.

The channel cuvette arrangement also provided a test pattern to check the effectiveness of ratioing in rejecting pathlength and excitation nonuniformities. For example, in an image encompassing both channel and non-channel areas (indicator and Ca concentration uniform), where the blank corrected fluorescence levels were different by a factor of two, the ratio varied, by less than 1.5% Similar rejection ratios were measured when the excitation was made non-uniform by closing down the field stop iris of the epilluminator while in an off-center position.

*RESULTS*

Because the imaging method is rather new, a large number of experiments were performed to investigate whether the indicator and the apparatus give valid readings of intracellular $Ca^{2+}$ changes. Figure 1 illustrates one such experiment. It is well known that in most central neurons, depolarization brings about $Ca^{2+}$ entry (22). When the depolarization is sufficient to trigger action potentials, the Ca influx is greater. In the neural network which exists on the culture plate after several days *in vitro*, elevating potassium in the bathing medium from 4.7 mM to 25 mM is sufficient to cause intense neuron firing.

Figure 1A shows a differential interference contrast (DIC) picture of a large bipolar neuron grown in culture for 26 days. An extra cellular patch recording electrode was sealed on to the soma membrane during the experiment. Seal resistances on the order of $10^9$ ohm are sufficient to allow action potentials and at least a fraction of incoming synaptic input to be monitored in these preparations. The cell shown was receiving measurable excitatory synaptic input at a mean rate of approximately 5 Hz. Most inputs were subthreshold and the cell fired action potentials irregularly at a mean rate of less than 0.5 Hz in normal Krebs saline.

Figure 1B shows the fluorescence image of this neuron using 340 nm excitation. Background and cell light levels for this image, which were in the low range of those encountered, are described in footnote 1. Figure 1C-F shows ratio images (340/380) of the neuron under different conditions. Figure 1C shows the fluorescence ratio when the cell is in normal Krebs saline. Ratio values have been coded in false color, with increasing values of the ratio shifting from blue to red. It will be noted that the nuclear region of the soma showed the smallest ratio in the resting state (.65 corresponding to 60 nM) while the non-nuclear regions were higher, approximately 0.9 (110 nM). Such non-uniformities between nuclear region and other parts of the cell were the rule rather than the exception in hundreds of cells examined. At this point it is unclear whether the difference in ratio reflects a true difference in free calcium or modified dye characteristics in the nucleus. Others (28), having measured *higher* ratios in nuclei of smooth muscle cells, have concluded that the ratio differences reflect true differences in free calcium. At the time of recording, the cell was receiving excitatory input as described above and the region of high $Ca^{2+}$ in the lower neurite may possibly be an input site. Exposing the culture plate to high potassium Krebs saline (25 mM) caused high frequency action potential firing (~ 10 Hz) and a sharp increase in the ratio shown in Fig. 1D. The record of Fig. 1D was made approximately 30 s after the saline change. The increase continued for the duration of the high K exposure (2 min). Using the conversion formula given in the methods section, the soma ratio predicts a $Ca^{2+}$ level of approximately 300 nM during the K exposure, up from the 100 nM levels of non-nuclear cytoplasm in 1C. The increase was not uniform throughout the cell, the proximal neurites showing a smaller increase than the soma. Graubard and Ross (9)

have also noted smaller axonal signals from Ca indicator arsenazo III in crustacean neurons during depolarization. A small portion of the soma-neurite difference noted in the present experiment, however, is artifact arising from the relatively faint fluorescence of flat glial cells in the vicinity of, and presumably also underneath the neuron. This background is usually less than 5% of the soma fluorescence, and 10-15% at the proximal neurites because of the different thicknesses of the structures. This background fluorescence is relatively insensitive to high K and therefore exists as a constant term in both numerator and denominator of a ratio. The ratio values for a more dimly fluorescent region will then be closer to unity than will a bright one. This background is not a problem in very young cultures in which cells have not overgrown the coverslip but these cells are poorly excitable (1). Reexposing the plate to normal Krebs saline led to a complete recovery of the initial ratio levels within 1.5 min. Figure 1E shows the ratio 10 minutes later, and illustrates both this recovery, and the reproducibility generally encountered in experiments. The record of Fig. 1F was made just after the patch recording electrode was pushed against the soma. The extracellular electrode monitored high frequency action potential discharge probably indicative of mechanical pressure upon the cell. The fluorescence ratio became correspondingly larger (predicted $Ca^{2+} > 600$ nM). In other experiments where tetrodotoxin (0.3 μM) was used to block spontaneous spike activity, a decrease in the fluorescence ratio was noted. The Ca channel blocker nifedipine also caused a decrease in the ratio in a subpopulation of the cells examined.

Growing cell processes were studied in cells no more than 24 hours old that had been plated at approximately one-third the normal density, facilitating the identification of cell processes with their appropriate cell bodies. Coverslips with cells were transferred from the incubator to the microscope stage and equilibrated for 10 to 15 minutes in slowly flowing culture media at 34-36 degrees C equilibrated with a 90:10% air:$CO_2$ mixture. The coverslip was then scanned for cells or groups of cells that were extending processes. After examining several hundred cell tips, both by direct inspection and by time lapse imaging, it became possible to discriminate between processes that were extending and those that had stalled with a reasonably short inspection. The extension rate of filopodia was generally less than 0.7 μm/min in the recording chamber and uninterrupted activity

was seldom seen for more than 15 minutes. The intermittent nature of activity was possibly a result of the perfusion system which may have dissipated unidentified trophic agents in the medium. Extending processes were often bent toward the direction of flow.

Figure 2A shows a field of four cells as a DIC image (left) and a fluorescence ratio image (right) and illustrates several features usually seen in cultures of this age (20 hours or younger). In the ratio image, displayed on a gray scale, two of the cells are very bright (high Ca), one is about neutral and one very dark (low Ca). The three cells on the left had sent out processes while the fourth (upper right) remained rounded as when plated. The two lower cells were in an active growing stage at the time of observation while the upper left cell had essentially stalled. The fluorescence ratios (and corresponding estimates of $Ca^{2+}$) of the different cells varied from 0.65 (50 nM) in the upper right cell to 3.6 (approximately 1 μM) in the lower left hand cell. The centrally located cell showed a ratio of 2.3 (440 nM) in the soma with less than 20% variation in the processes sprouting from it. The ratio in the soma of the upper right cell was 1.1 (140 nM) with the process about 30% larger although this difference does not show up at the display scale used here. After the records of Fig. 2A were made, the central cell was examined at higher magnification for an extended period (pixel binning in the camera was discontinued giving a 2X enlargement). Figure 2B shows a series of DIC images of this cell taken at 4 minute intervals. It is clear that the cell is growing out at both ends, top and bottom, and sprouting side processes as well. Fluorescence images interspersed with these frames showed that the ratio remained around 2 for the duration of the observation period.

Such high free calcium levels as seen in the lower two cells of Fig. 2A does not represent an incompetence in the calcium regulatory system of the cells. Exposing such cells to a simple Hepes-buffered Krebs saline of identical calcium concentration caused a drop in the fluorescence ratio to levels generally below 0.8 within 5 min. Correlated with this decrease was an immediate arrest of growth and often a retraction or restriction of processes. Restoration of media reversed these changes in a small percentage of cases. The critical factors missing in the Krebs saline have not been ascertained. The important finding for this study is that the cells were able to reduce their

intracellular free Ca.

Figure 3 illustrates the common observation when an actively extending process had reached some distance from the soma. Figure 3A is a DIC image showing extended filapodia at the tip of an outgrowth. Figures 3B, C show the fluorescence images of the cell with 340 and 380 nm excitation. Figure 3D shows the ratio of the fluorescence images, the free Ca levels in the cell. The ratio in the extreme tip is between 1.45 and 1.65 and in the major portion of the cell body between 0.65 and 0.75. These ratios correspond approximately to free Ca levels in the tip of 230-270 nM and 50-70 nM in the cell body. It will be noted that there are regions of intermediate and lower levels along the process. During the course of a 25 min observation on this cell, the pattern of extension shifted from one where only the extreme upper part of the tip was involved to the one shown in the figure where most of the tip had sprouted filopodia. The region of localized high Ca spread concurrently with this change. Again substitution of Krebs saline for growth media caused a rapid drop in the high Ca regions. The free Ca levels of stalled growth cones were within 10% of the cell body whether all filopodia had been retracted or not.

Table I presents a summary of the observations categorized according to cell extension activity, rapid, slow, stationary. The rapidly extending category includes cells that were sprouting very near the soma and had rather uniformly high calcium levels and those where substantial processes had already been put down and extension was occuring at the end of these. In the latter cases (Fig. 3) free calcium was only high at the extending tip and a large intracellular gradient of free calcium existed in the cell. The number of cells scored in the intermediate category, slowly extending, was relatively small primarily because it was impractical to spend the time required to decide that the cell was doing anything at all. Many more cells than the 16 noted were observed at intermediate free calcium levels. The great majority of these cells had grown substantial processes at the time of observation. Stationary cells vastly outnumber the active ones in part because scoring has been done over a population where the proper growing conditions in the recording chamber were being worked out.

*DISCUSSION*

The results show a clear correlation between cell growth and high internal free calcium. During stationary periods or when processes were observed to retract, calcium levels were much lower and relatively uniform. In more mature cultures (2-5 days) fluorescence ratios greater than 0.7-0.9 were almost never observed. At later stages where spontaneous electrical activity was present the ratio again tended upward in some neurons (as in Fig. 1) but almost never near the 2.0 level unless the cells were stimulated by high K or other means. The observations on growing cells reported are in agreement with studies using electrical techniques in other preparations that have shown a calcium influx into growing tips via either Ca spikes (10, 14, 18) or a mechanism that carries steady calcium current measurable by external vibrating probe electrodes (8). It was noted in the vibrating probe studies that currents were not generated by stationary growth cones, only those in an active growth state. With the possible exception of cells used in the vibrating probe study, the electrical studies have been carried out on cells that had well expressed ion channel populations, being either cell lines or regenerating tissue. An extensive series of electrical measurements made on these diencephalon have shown them to be electrically inexcitable for the first 10 hours after plating. Between 10 and 24 hours a small fraction of the cells, less than 10%, generated small spike potentials upon depolarization, but these were sodium dependent and blocked by tetrodotoxin (1). Thus the electrophysiology of these developing cells is fundamentally different from most other systems studied. Approximately 90% of the cells that survive the dissociation and plating procedures had undergone final mitosis within the 24 hour period proceeding isolation.

Nifedipine (5 μm), a Ca channel blocker in many nerve cultures, was ineffective in stopping growth or reducing high calcium levels where they were present. Tetrodotoxin was also ineffective. The inorganic Ca channel blockers, Cd and Mn, disrupted cell growth but had interactions with the intracellular fura-2 (and with quin 2) in these and other culture cells that made the experiments uninterpretable (Connor and Hockberger, in preparation). Therefore, at present, the mechanism responsible for maintaining the high free calcium levels is not understood.

The existence over periods of minutes of Ca gradients within a cell such as in Fig. 3 would seem to require the presence of localized Ca influx at the growing tip with efflux or storage predominating over the rest of the cell, or else internal compartmentalization as in vesicles or organelles at the growing tip. It is known from other studies that fura-2 does become trapped in intracellular compartments (3, 28) as well as the cytoplasm. In the cells studied here, it has not been uncommon to observe large vesicles in the soma with fluorescence ratios different from the cytoplasm, both higher and lower. These were noted with either fura-2 or quin 2 and were usually prominent only after the cells had been removed from the culture medium and exposed to Krebs saline for extended periods. Thus the possibility exists that the locally high Ca in the growing tips is trapped in vesicles too small and densely packed to be resolved, even using a 100X objective as was the case in some experiments. It should be emphasized though that the content of such vesicles was very sensitive to the extracellular environment because the gradients were largely abolished within 2 to 3 min of the withdrawal of media and its substitution by Krebs saline. Unlike some amphibian cells (13) these mammalian CNS cells do not sprout processes to any normal degree or even survive well in Ca-free media (Tseng and Connor, unpublished).

The data shown here have been of fura-2 fluorescence. Earlier experiments (5, 6) used quin 2 and showed the same essential features as the fura-2 data with the interesting exception that the regions of high calcium were never so localized. This difference is not surprising given that the loading level of quin 2 was about 10X that of fura-2 in the cells and the higher level of calcium buffer would tend to damp out spatial gradients. These techniques can be effectively applied to study a variety of physiological questions, for example, regional distributions of $Ca^{2+}$ channels or transport into cells, dynamic changes in $Ca^{2+}$ levels in response to neuroactive substances, and development of effective synaptic connections.

Software for the imaging system was written by K. L. McMillan. Thanks is expressed to A. J. Tyson for advice on CCD cameras, to Z. Ahmed, P. E. Hockberger and H. Y. Tseng for assistance in tissue culture preparations, to G. Blonder for fabricating calibration slides and to Simon Levy for checking standardization solutions with a Ca-electrode. Partial support provided by AFOSR F496 20-85-C-0009.

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## FIGURE CAPTIONS

Fig. 1. A: DIC (Normarski) image of bipolar neuron grown 26 days in culture. Small round cell (lower right) failed to trap indicator. B: Fluorescence of trapped indicator (340 nm excitation) illustrating relative intensities in various regions of the cell and background level (see footnote). Upper process could not be displayed without severely distorting the relative brightness of soma. C: Fluorescence ratio (340/380 nm) of cell bathed in Krebs saline (4.7 mM K). "Haloing" is due to out of focus fluorescence and is most severe at soma where cell is thickest. D: Fluorescence ratio of cell exposed to high K saline (25 mM) for 30s. Red shifted false color indicates higher Ca. Extreme levels of blue and red correspond to ratios of 0.4 and 2.0. E: Ratio 10 min. after return to normal saline. F: Ratio image after movement of patch electrode on soma that caused a transient high rate of firing. Dark pixels in the soma are off scale (ratio > 2). Calibration bar 10 μm.

Fig. 2. A. Left, DIC image of a field of 4 cells plated 16 hr. previously. Right, grey scale ratio image, 340/380, of same field. Ratio values given in text. Cell in upper right is oversized in ratio image both because of its relative thickness (out of focus light) and the necessity of emphasizing contrast at around unity which was done to keep upper left cell from disappearing into the background. The ratio values in the halo are approximately the same as from the center of the cell. Background grey level was arbitrarily chosen for maximum visibility of cells. B. Enlarged DIC images of center cell taken at approximately 4 min. intervals illustrating growth rate of high Ca cells. Calibration bar: 20 μm in A, 10 μm in B.

Fig. 3. A. DIC image of cell 20 hrs in culture sending out filopodia (upper right). B. Fura-2 fluorescence, 340 nm excitation. C. Fluorescence, 380 nm excitation. D. Ratio of fluorescence images displayed in false color map. Ratio value of color and corresponding calcium level given in text. Calibration bar, 5 μm.

## TABLE CAPTION

Table I Compilation of data showing correlation of $Ca^{2+}$ levels with extension rate (see text for details).

Table I

| CELL STATE | NUMBER OBSERVED | FLUORESCENCE RATIO AT ACTIVE REGION | ESTIMATED FREE $Ca^{2+}$ (nM) |
|---|---|---|---|
| EXTENDING AT 0.3 μm/min OR MORE | 34 | 1.4 – 3.5 | 200 – 1000 |
| RANDOM MOVEMENT OR SLOW OUTGROWTH | 16 | 0.8 – 1.4 | 70 – 200 |
| PREEXTENSION STAGE OR STALLED | > 150 | 0.6 – 0.8 | 30 – 70 |

**Footnote 1**

Light intensities are expressed in analog-to-digital units (ADU). For the chip used 1 ADU corresponds to approximately 20 electrons. At −40°C camera dark noise (pixel + readout) was 20 ADU/pixel ± 0.5 (computed from a center field block of 30×30 superpixels). With saline on a blank coverslip these values were 21 +1 0.5 for 360 nm. Indicator fluorescence typically ranged from 20 to 100 ADU/pixel in the cell somata (dark corrected) depending upon thickness and loading and 7.5 to 13 in the larger processes (340 nm excitation), well above pixel variation. (Pixel to pixel variation (~0.5 ADU) is the critical parameter since dark noise can be subtracted, leaving a prefectly good image if the variation is small. For 360 nm excitation the corresponding values were 28 to 59 ADU/pixel in somata and 10 to 14 in processes. Intrinsic fluorescence of these cells was less than 3% of the total. Lamp intensity at 340 nm was not sufficient to generate an intrinsic signal much above the camera noise. At 360 nm excitation, removing the neutral density filters 1.5 O.D. gave dark corrected soma intensities ranging from 20 to 50% of indicator fluorescence measured in comparable cells with the neutral density filter in place.

DEVELOP. BRAIN RESEARCH (in press)

Title: Development of Membrane Currents in Fetal Rat Neurons

Authors: Z. Ahmed, J.A. Connor, D. Tank and R. Fellows

Running Title: Development of membrane currents in fetal rat neurons

Number of pages: 22

Number of figures: 7

Number of tables: 1

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Keywords: neuronal differentiation, neuronal development, membrane currents, serum-free culture, development of excitability, diencephalic neurons

ABSTRACT

Studies on developing neurons from different animal sources have shown a wide variation in the order in which ionic channels appear in the membrane. Using the whole cell gigaseal voltage clamp technique we have investigated the expression of voltage dependent ionic currents in dissociated primary cultures of fetal rat (E17) diencephalic neurons grown in a serum-free defined medium. At this date we have been able to characterize at least two populations of neurons with different developmental properties. One class of cells first expressed a transient inward current after 10-24 hr in culture. These transient, all-or-none, inward currents arose at the cell neurites where the membrane voltage was not under space-clamp. The all-or-none inward current coud be reversibly blocked by either 1μM tetrodotoxin (TTX), 1 mM cobalt or cadmium. No appreciable outward current was present in the cells at this stage. With further maturation the magnitude of the all-or-none current was increased. After about 6 days in culture these cells developed a large, inward current whose activation was graded with voltage. At this stage a transient outward current was also present. In external cobalt the inward current showed a small reduction in amplitude but an increase in duration. The transient outward current was abolished. In TTX more than 85% of the inward current was blocked but the outward transient remained. The outward transient appeared to consist of both an A-current and a calcium activated transient. The second population of neurons expressed a voltage dependent outward current in the soma after 20 hr in culture. After 3 days these cells expressed a voltage-dependent TTX-sensitive, cobalt insensitive inward current in the soma which showed the typical I-V relationship observed in other vertebrate neurons.

## INTRODUCTION

An important step in the overall process of neuronal cytodifferentiation is the expression of membrane ionic channels underlying active currents. Previous studies on developing neurons from various animal sources have shown that while there is no fixed pattern of expression of these ionic channels, some common features are observed (Baccaglini and Spitzer, 1977; Spitzer, 1979; Goodman and Spitzer, 1979; Willard, 1980; Goodman et al., 1980; Bader et al., 1983; Ahmed et al., 1983; Peacock and Walker, 1983). While differences in the sequence of expression of inward membrane current have been observed even in vertebrate neurons, there continues to be a controversy over whether the ionic dependence of the action potential undergoes developmental change. Also, differences in the sites of expression of ionic channels have been observed in neurons from different sources (Llinas and Sugimori, 1980a,b; Goodman and Spitzer, 1979; Willard, 1980; Smith, 1983). As a result, there is considerable uncertainty about the specific mechanisms of expression of electrical excitability in developing neurons. Also, knowledge of the expression of electrical excitability in developing mammalian brain neuron is still fragmentary or lacking.

In this paper we describe, and attempt to classify, the properties of the expression of voltage-dependent membrane ionic currents in dissociated culture of rat diencephalic neurons grown in a serum-free defined medium. A preliminary report of this work has been presented (Ahmed et al., 1984).

## METHODS

Brains were obtained from 17 day old rat embryos under sterile conditions. Following the removal of meninges and blood vessels, the cerebrum was discarded. The diencephalon was dissected out by a transverse cut rostral to the tectum. Tissue pooled from 8-10 fetuses was dissociated

enzymatically by a procedure described previously (Ahmed et al., 1983) and dissociated cells were cultured in the serum-free defined medium described by Ahmed et al. (1983) with the following modifications: streptomycin, penicillin A, fungizone and HEPES were left out, and the concentration of KCl was reduced from 25 to 5.4 mM. Cells were grown in 24-well Falcon dishes on 12mm (No. 1) coverslips precoated with poly-D-lysine.

At the time of plating dissociated cells were well separated and evenly distributed throughout the culture dish. Cells were spherical, 10-15 microns in diameter and fewer than 5% of cells having rudementary processes no more than twice the cell diameters in length. Cells began to extend processes within 1 hour after plating and by 3 hours a majority of the cells had extended processes up to several times the cell diameter. By 24 hours in culture about 85% of the cells were rounded and exhibited processes; these cells could be divided into two groups. One group had long processes which were generally highly branched at a distance from the perikaryon. These cells made up about 65% of the total and were presumed to be neurons based on morphology (Mirsky and Thompson, 1975) and the ability to bind tetanus toxin (Ahmed et al., 1983). Cells of the second group had many shorter, thinner processes which branched frequently near the cell body. These cells made up about 20% of the total and were identified as astroglia on the basis of morphology and immunocytochemical staining of glial fibrillary acidic protein (Ahmed et al., 1983). The remaining 15% of the cells were large and generally flat, with few or no processes, consistent with the appearance of oligodendroglia and fibroblasts (Raff et al., 1979). These morphological features of the rounded and flat cells were retained throughout the culture period. Since unambiguous classification into neuron versus astroglia was difficult during electrophysiological experiments, recordings were made from rounded cells with one or more processes chosen at random.

For electrophysiological recording, a coverslip containing cells was removed from the culture dish and placed in a special chamber on the fixed stage of a Ziess inverted microscope equipped with phase contrast optics. The chamber was continuously perfused at 0.1 ml/min to a depth of approximately 2 mm with a solution containing 145 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$ and 10 mM Hepes, pH 7.4 (control saline). The chamber temperature was maintained at 37°C by a local heating circuit.

Patch pipettes were fabricated from WPI 150-6 capillary glass and filled with 140 mM potassium, 2 mM $MgCl_2$ and 10 mM HEPES pH 7.2. In some experiments 1-100 μm EGTA was added to the pipette solution. The presence of EGTA stabilized whole-cell recordings in these cultures. An understanding of this dependence is, at present, lacking. The pipette was placed on the head-stage of a List L/M EPC-5 (1 GΩ or 10 GΩ feedback resistor) patch clamp unit and was positioned with a Narashigi hydraulic drive micromanipulator. Usually pipettes with resistance values of 3-7 MΩ in control saline were used. Following the establishment of a gigaseal, pulses of suction were applied to the pipette interior to establish tight seal whole cell recording mode. Membrane currents at later developmental stages (3 days on) were sufficiently large to introduce series resistance based artifacts in the current records (Hamill et al., 1981). Because we were also faced with additional space clamp artifacts due to cell geometry, we did not regard electronic compensation of series resistance useful and thus avoided extensive quantitative interpretation of the whole cell current data. Data were recorded on a Vetter FM-tape recorder, and photographed from an oscilloscope. In some current traces the capacitive transients have been blocked out in the figures for the purposes of clarity. All experimental solutions were filtered with 0.2 μm filters and cells were rinsed 3-5 times with the filtered solution.

## RESULTS

Electrophysiological properties of neurons were examined in 10 hour to 11 day old cultures. The resting membrane potential was measured from the pipette potential, under current clamp at zero pipette current after establishing the tightseal whole cell recording mode (Hamill et al., 1981). There was no significant change in the distribution of cell resting potentials during maturation from 10 hours to 11 days in culture; the values of the resting potential were distributed in the range between -45 mV and -70 mV. Cell input resistance was calculated from the change in membrane voltage, from a holding potential of -60 mV, due to an applied hyperpolarizing current of 1-5 pA amplitude and 500 msec duration. The input resistance of cells in 10 hour to 9 day old cultures ranged between 6 and 10.5 GΩ , values similar to those observed in bovine chromaffin cells (Fenwick et al., 1982). Membrane currents were recorded by applying brief depolarizing voltage steps superposed upon a holding potential set at -40 mV, -60 mV or -80 mV.

Electrophysiological properties were observed to vary among cells at any particular age of the culture. This is probably due to the presence of a heterogeneous population of cells in the culture, since the embryonic rat diencephalon contains more than one population of neuronal and non-neuronal cells. In order to study the development of membrane excitability under these conditions of cellular heterogeneity, we have investigated populations of cells in chronological sequence and have grouped the cells at each developmental stage according to their observed membrane properties (see Discussion, Table I). In the following and in Table I, the classes are enumerated with Roman numerals and letters. Groups with broadly different electrophysiological properties have different Roman numerals (eg., IA, IIA).

Groups which are different but appear to be developmentally related have different letters (e.g., IIA, IIB).

Cells examined after 10 to 30 hours in culture can be grouped into three populations. The first group of cells (Group I) did not show any regenerative currents. There was no change in the current traces when the holding potential was hyperpolarized to -80 mV or depolarized to -40 mV, indicating that the lack of any voltage-dependent current is probably not due to the inactivation of channels. In the second type of cells (Group IIA) application of depolarizing voltage pulses activated only voltage-dependent outward currents. A typical series of membrane currents recorded from a 24 hour old neuron is shown in fig IA(i). Bath application of 20 mM tetraethylammonium (TEA), which blocks voltage-dependent $K^+$-channels in a wide variety of excitable cells (c.f. Stanfield, 1983), blocked the outward current (fig. IAii). Addition of 1 mM cobalt or cadmium did not affect this current. The current-voltage relationship of this neuron in control saline (filled circle) and in saline containing 20 mM TEA (open circle) is shown in fig. IB. The outward current began to activate at approximately -35 mV and exhibited the voltage-dependent increase in magnitude characteristic of nearly all other neurons (Hodgkin et al., 1952). The third group of cells (Group IIIA) in 10-30 hour old cultures produced time invariant currents for depolarizing voltage steps up to +35 mV from a holding potential of -60 mV (fig. 2A). The magnitude of these currents changed linearly with voltage (fig. 2C). Presence of external TEA (20 mM) or cobalt (1 mM) had no effect on these currents. Although these cells did not produce any other voltage- or time-dependent currents (other than the leakage current) for depolarizing steps up to about +35 mV applied from a holding potential of -60 mV, application of depolarizing voltage steps from a holding potential of -80 mV evoked a transient outward current (Fig. 2B). The amplitude of the transient

current increased upon stepping to more positive command potentials. At holding potentials equal to or more positive than -60 mV, the transient outward current was completely inactivated (Fig. 2A). The addition of 20 mM TEA did not significantly affect this current. These characteristics of the transient outward current are similar to $I_A$ of molluscan neurons (Hagiwara et al., 1961; Connor and Stevens, 1971). A similar transient $K^+$-current was observed in cultured rat hippocampal and mouse spinal neurons (Segal et al., 1984). In addition to the transient outward current, this third group of neurons (group IIIA) exhibited delayed all-or-none inward currents for larger depolarizing voltage steps to +40 mV and above (see Fig. 3). Increasing the voltage step size reduced the delay (or latency) between the onset of the voltage step and the onset of all-or-none current. In most cases multiple all-or-none currents were observed (fig. 3). The amplitude of the currents was reduced only slightly by increasing the depolarizing step size by 40 mV. It is likely that the all-or-none currents arise at electrically distant neurites where membrane voltage is not under adequate space-clamp. Similar all-or-none currents have been observed in the neurites of neuroblastoma cells, where the cell body was under two microelectrode voltage clamp (Moolenaar and Spector, 1978) and are a well described phenomenon in molluscan neurons (Kado, 1973; Connor, 1977).

The all-or-none current could be reversibly blocked by 1 μM tetrodotoxin (TTX) (fig. 3B), or 1 mM cobalt or cadmium (fig. 3D), in the bathing solution. Replacement of external $Na^+$ with tetramethylammonium (TMA) also blocked the all-or-none currents, suggesting that the channels involved in the all-or-none current is probably $Na^+$-selective. We are not aware of other instances where cobalt or cadmium blocks a $Na^+$ current at concentrations typically used to block $Ca^{2+}$-currents.

Based on similar properties, cells examined after 42 to 54 hours in culture can also be divided into the same three groups. Cells examined after 68 to 78 hours in culture showed both quantitative and qualitative differences in their membrane properties. A large proportion of these cells had properties similar to those of Group IIIA. However, the amplitude of the depolarizing voltage step necessary to generate the all-or-none currents reduced by about 40 mV from that of the 10-30 hour old cells. Also, the amplitude of the all-or-none current increased by a factor of 2 or more. Fig. 4 shows voltage step induced multiple all-or-none currents in a 3 day old cultured neuron. The all-or-none currents retained their pharmacological properties in that they could be reversibly blocked by 1 μM TTX (fig. 4B), 1 mM cobalt or cadmium (fig. 4D). At this age of the culture, and at later stages, cells with only voltage-dependent outward currents (group IIA) were absent. However, two populations of cells with other membrane currents in addition to those of group IIA and IIIA were observed. Cells of one classification (group IIIB) developed large inward current whose activation was graded with voltage and a transient outward current that did not require prehyperpolarization. Those of second classification (group IIB) exhibited voltage-dependent inward current of somewhat different properties and a delayed rectifying current. Cells of both these groups were present up to 11 days in culture. Fig 5A shows the membrane currents recorded in control saline from a 5 day old neuron of group IIIB for a command voltage step to -15 mV from a holding potential of -60 mV. The large inward current and the transient outward current characterized the neurons of this group after 3 to 11 days in culture. In the presence of external cobalt or cadmium (1 mM), the inward current showed 17-21% reduction in amplitude, and the duration increased by a factor of 1.7 to 2.1 (fig. 5B). Also the transient outward current was completely abolished. In 1 μM TTX, 80-85% of the inward current

was blocked but the transient outward current remained (fig.5C). Replacement of the external $Na^+$ with TMA also blocked 80-85% of the inward current and only slightly affected the transient outward current. These results suggest that roughly 80% of the inward current of cells of group IIIB is carried by $Na^+$, and the transient outward current is probably a calcium activated current. All these values are of limited accuracy due to the problems of cell geometry and series resistance mentioned in the Methods section. The broadening of the inward current in external solution containing 1 mM cobalt or cadmium (fig. 5B) suggests that in control saline the duration of the inward current is small because there is nearly simultaneous activation of the outward current. An intracellular $Ca^{2+}$-dependent, fast $K^+$-current has been observed in bullfrog sympathetic neuron (Adams et al., 1982). These cells (group IIIB) showed the presence of voltage-dependent transient outward current ($I_A$, similar to fig 2) in presence of 1 μM TTX and 1 mM cobalt when the membrane potential was held at -80 mV. Also, in a number of cells, longer duration voltage steps (50 msec or more) produced multiple all-or-none currents in addition to the large inward current. These similarities of group IIIB cells with those of group IIIA cells suggest a possible developmental relationship between these groups (see Discussion).

A series of membrane currents recorded from a 7-day old neuron of group IIIB is shown in fig. 6A. Depolarizing voltage steps were applied from a holding potential of -60 mV. The latency between the onset of the voltage pulse and the onset of the inward current, which is quite pronounced for smaller voltage steps, decreases with increasing step size. Fig 6B shows the current voltage relationship of the peak inward current of the neuron of fig 6A (□) of peak value of the transient outward current (o), and value of the outward current at the termination of the voltage pulse (•) The fast inward

current (□) is activated at membrane voltage slightly above -40 mV, a value similar to the activation of the outward currents. The reversal potential of the fast inward current is about +35 mV, at which the peak I-V curve crosses the extrapolated leakage current. The I-V relationship of the peak transient outward current (o) reaches its maximum value at -10 mV, where the peak inward current also reaches its maximum value. The plateau outward current (•) shows the linear I-V relationship typical of leakage currents and indicates that there is little or no delayed rectifier current.

A series of membrane currents recorded from a 6 day old neuron of group IIB is shown in fig 7A. Depolarizing voltage steps were applied from a holding potential set at -60 mV. Hyperpolarizing and small depolarizing voltage steps in the range between -100 mV to -40 mV produced small time invariant leakage currents, whose magnitude changed linearly with voltage. As the voltage step-size was increased, inward current was initiated. This current revealed voltage-dependent activation and inactivation with a relatively fast time course. The inward current was completely abolished in the presence of 1 μM TTX without affecting the outward or the leakage currents. Essentially the same result was obtained in $Na^+$-free solution, Na-replaced with TMA. The presence of 1 mM cobalt or cadmium did not affect the inward current. The outward current began to activate at membrane voltages around -35 mV. The outward current could be blocked by 20 mM TEA but was unaffected in presence of 1 mM cobalt or cadmium. Fig. 7B shows the current voltage relationship of the peak inward current (o) and steady-state outward current (•) in control saline. The peak inward current reached its maximum value rather precipitately at around -30 mV. The reversal potential of the peak inward current was at around +35 mV (peak I-V curve crosses the extrapolated leakage current). The outward current exhibited a voltage-dependent S-shaped rise, seen in other neurons (Moolenaar and Spector 1978;

Bader et al., 1983). The properties of the outward current of group IIB cells were very similar to those of group IIA (see discussion).

In addition to these groups of cells, 2 out of a total of 34 cells examined after 9 to 11 days in culture (not included in table I) exhibited large voltage dependent fast and slow inward current. Both the fast and the slow components of the inward current showed voltage dependency. The fast current was insensitive to 1 mM cobalt or cadmium, but was completely blocked in presence of 1 μM TTX. The slow inward current was insensitive to TTX, but was blocked in presence of 1 mM cobalt or cadmium. Because the frequency of occurrence of these cells was very low, they are not included in table I.

## DISCUSSION

The purpose of this investigation has been to examine the expression of ionic currents during development of dissociated rat diencephalic neurons grown in a serum-free culture medium. The process of dissociation involves radical disruption of the tissue and separation of tissue into individual cells. The freshly dissociated cells are almost completely devoid of processes, and appear to be undifferentiated neurons, i.e., neuroblasts. It is generally considered that differentiated or partially differentiated cells with any significant extension of processes do not survive the dissociation procedure (Dichter 1983). In a separate study, we have found that more than 95% of the cells dissociated from 17 day old fetal rat diencephalon are in $G_1$-phase of the cell cycle and remain in $G_1$ phase throughout the culture period. About 40% of the cells have undergone mitosis within a period of 24 hours prior to the dissociation (Ahmed and Fellows, in preparation). Also, in serum-free defined medium there is no further proliferation of neuronal cells (Ahmed et al, 1983). Therefore, it is most likely that the membrane

properties that were observed in this study are those expressed by differentiating (or developing) neurons of the diencephalon. Although the dissociated diencephalic cells are fairly homogenous in terms of their cell cycle position, these cultures contain more than one population of neurons. Since neurons of a number of nuclei of the rat diencephalon are formed during the 16-17th day of gestation (Altman and Bayer 1978; Altman and Bayer, 1979), and, as undifferentiated neurons would be able to survive the dissociation procedure, it is likely that more than one population of neurons is present in the culture. The observation of cells with differing aggregates of membrane propoerties is probably a simple reflection of the surviving cell types.

Table I lists five different classes of membrane properties observed in 10 hour to 11 day old cultures. Group I cells, which did not show any voltage- or time-dependent current, are not of much interest in the present study. Their relative frequency of occurrence is high during the early periods of culture, and decreases with time. It is likely that in younger cultures (10-45 hours) some of the cells have not yet expressed any membrane currents. Also some of the recorded cells may be non-neuronal because, at this stage of the culture neuronal cells are not easily recognized based on their morphology. In older cultures, our selection procedure was less susceptible to this problem since neuronal cells are more easily recognized based on their morphology (Mirsky and Thompson 1975). This may be, at least partially, responsible for the decrease in the frequency of occurrence of Group I cells with age of the culture.

Cells of Group IIA are present only during the initial period of culture. Their characteristic membrane property is the presence of voltage dependent, TEA sensitive outward current. The voltage dependency of the outward current indicates that the ionic channels are probably located in

membrane at or near the cell body. Cells of group IIB also exhibited a similar outward current along with a TTX-sensitive, cobalt/cadmium-insensitive inward current, graded with applied voltage. The inward current in these cells is carried by $Na^+$ and the channels responsible for carrying the bulk of the current are probably located in or near the soma. Cells of Group IIB were only seen after 3 days in culture. Because of the similar properties of the outward current and the time sequence in which the cells of group IIA and IIB are seen in culture, it is likely that the cells of group IIA and IIB belong to the same class of neuron, the cells of group IIB being a more differentiated version of the cells of group IIA. Studies with cultured avian mesencephalic neurons have shown that the voltage dependent $K^+$- and leakage-currents are expressed first, followed by the expression of $Na^+$ and in some cells $Ca^{2+}$ currents (Bader et al., 1983). A progression from Group IIA to Group IIB is consistent with the sequence of expression observed in avian neurons.

Cells of group IIIA showed all-or-none currents generated at the neurites and a transient outward current similar to A-current (Connor and Stevens, 1971) generated in the cell body. Since the all-or-none currents were abolished by the addition of TTX or by the removal of external $Na^+$, they were carried by $Na^+$. However, the blockade of the all-or-none current by cobalt or cadmium raises a question about the nature of this early ionic channel. It is possible that the channels expressed early during development are $Na^+$ channels having some common structural feature to the $Ca^{++}$ channels. The existence of a structural feature common to both $Na^+$ and $Ca^{++}$ channels in neuroblastoma cells has been suggested (Romey and Lazdunski, 1982). Alternatively, the involvement of an autosynapse may also explain the cobalt or cadmium blockade. In any case, the data suggests that the early expressed

$Na^{++}$ channels are located in the neurites. Cells of Group IIIB also had some of the Group IIA membrane properties in addition to a large inward current generated in or near the cell body, and a $Ca^{2+}$-dependent transient outward current. About 85% of the inward soma current is carried by $Na^{+}$ and the remainder by $Ca^{++}$, both of which have the known pharmacological specificity. Again, because of the similarities in membrane properties of Group IIIA and IIIB cells and the time sequence in which the cells of group IIIA and IIIB appear in culture, it is most likely that cells of group IIIB are a more developed version of group IIIA cells. The sequence of expression of membrane current in group III cells resembles somewhat that of the developing neurons of grasshopper. In grasshopper neurons, $Na^{+}$-current is first expressed in the axons and, with further maturation of the neurons, currents are expressed in the cell body (Goodman and Spitzer, 1979; Goodman et al., 1980).

This study has demonstrated that at least two classes of neurons with different developmental properties, based on electrophysiological criteria, are present in dissociated primary culture of E17 fetal rat diencephalon. One class of cells first develops inward current in the neurites and subsequently in the cell body. The neurite inward current was largely carried by $Na^{+}$, but unexpectedly, both $Na^{+}$ and $Ca^{2+}$ channel blockers were able to block the current at early stages of development. At later stages the inward channels exhibited classical pharmacological specificity. The second class of neurons first expresses a voltage-dependent outward current in the soma and then expresses voltage-dependent inward $Na^{+}$ current in the soma. Both the inward and outward currents show classical pharmacological sensitivity. It should be noted that this study has demonstrated that the expression of ionic channels in the membrane of the rat diencephalic neuron can take place in the absence of all systemic hormones known to influence the

development of the brain. Since the serum-free defined medium does not contain any hormone other than insulin, which is necessary for the survival of these cells in culture (Ahmed et al., 1983), it provides an excellent opportunity for the investigation of basic mechanisms underlying neuronal differentiation.

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FIGURE LEGENDS

Fig. 1. A, Records of outward currents during depolarizations from a holding potential of -60 mV. Transmembrane potential is shown to the right of the current trace. (i) in control saline (ii) in saline containing 20 mM TEA. No EGTA in electrode. 24-hour old culture. B, Current-voltage relationship of the same neuron in control saline (o) and in saline containing 20 mM TEA (o). Currents are typical of Group IIA cells in 10-54 hour old cultures.

Fig. 2. A, Membrane currents during depolarizations from a holding potential of -60 mV in control saline. 40-hour old culture. B, Records of transient outward currents from a 36 hour old cultured neuron. Depolarizing steps were applied from a holding potential of -80 mV. Typical of Group IIIA neurons. C, Current-voltage relationship of the neuron in A. Currents measured near the termination of the voltage pulse.

Fig. 3. Multiple all-or-none neurite current recorded from a 36 hour old cultured neuron (Group IIIA). A, in control saline; B, in presence of 1 uM TTX; C, Reversal from TTX block; D, in presence of 1 mM cadmium. Voltage step to +40 mV from a holding potential of -60 mV.

Fig. 4. Multiple all-or-none neurite current recorded from a 3 day old cultured neuron (Group IIIA). A, in control saline; B, in presence of 1 uM TTX; C, reversal from TTX block; D, in presence of 1 mM cadmium. Voltage step to +5 mV from a holding potential of -60 mV.

Fig. 5. Membrane currents recorded from a 5 day old cultured neuron (Group IIIB). A, in control saline; B, in presence of 1 mM cadmium which eliminated the transient outward current; C, in presence of 1 uM TTX, which blocked most of the inward current. Voltage step to -15 mV from a holding potential of -60 mV.

Fig. 6. Membrane currents recorded from a 7-day old neuron (Group IIIB) in control saline. Command voltage is shown on the right of each current trace. B, Current-voltage relationship of the same neuron. Peak inward current (open squares), peak transient outward current (open circle) and plateau outward current (filled circle).

Fig. 7. A, Membrane currents recorded from a 6-day old cultured neuron (Group IIB) in control saline. Command voltage steps are shown on the right of each current trace. B, Current-voltage relationship of the same neuron. Peak inward current (open circle) and outward current (filled circle).

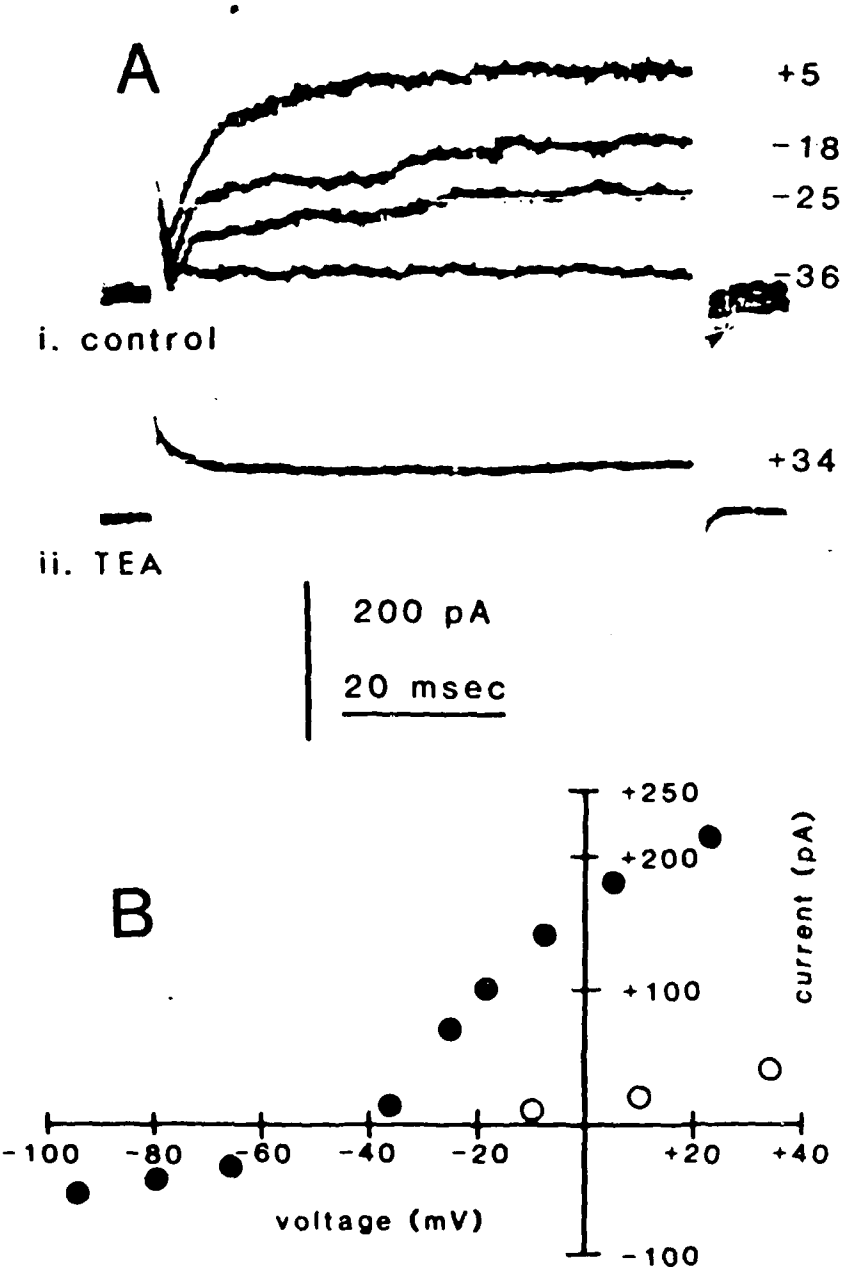
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Fig 1.

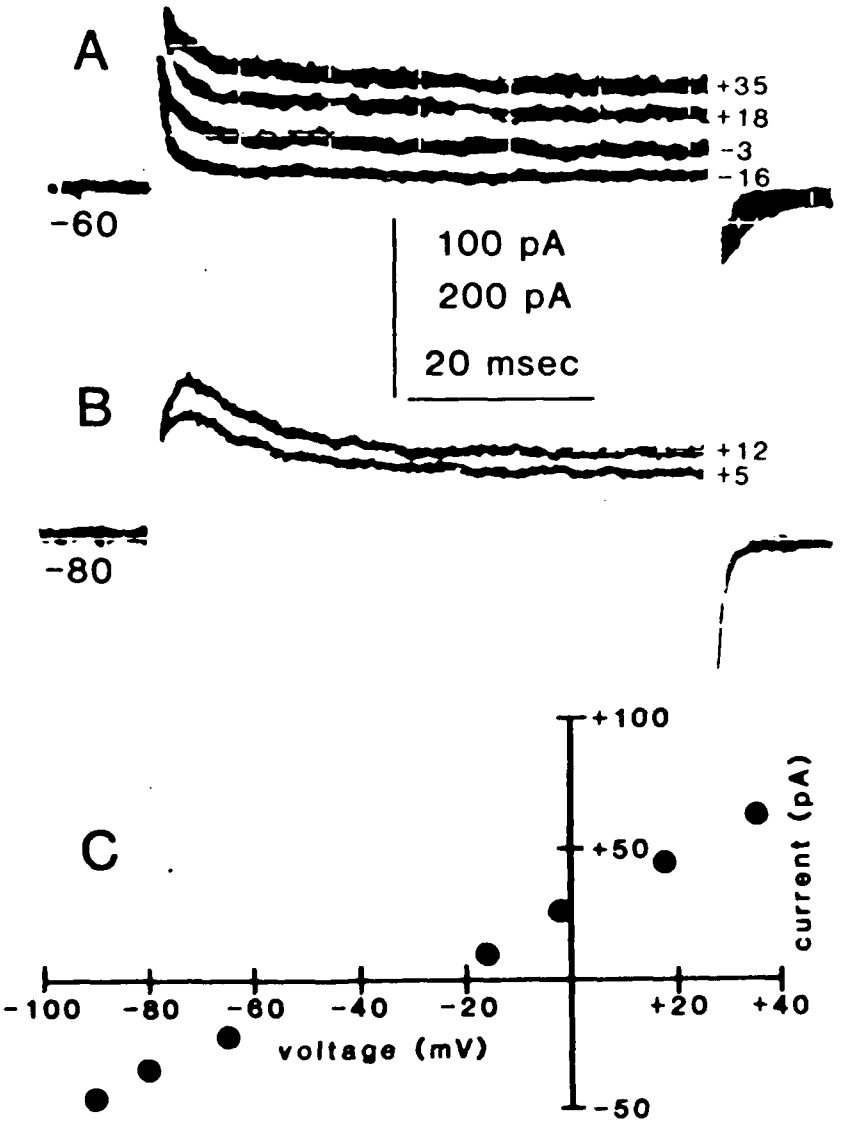
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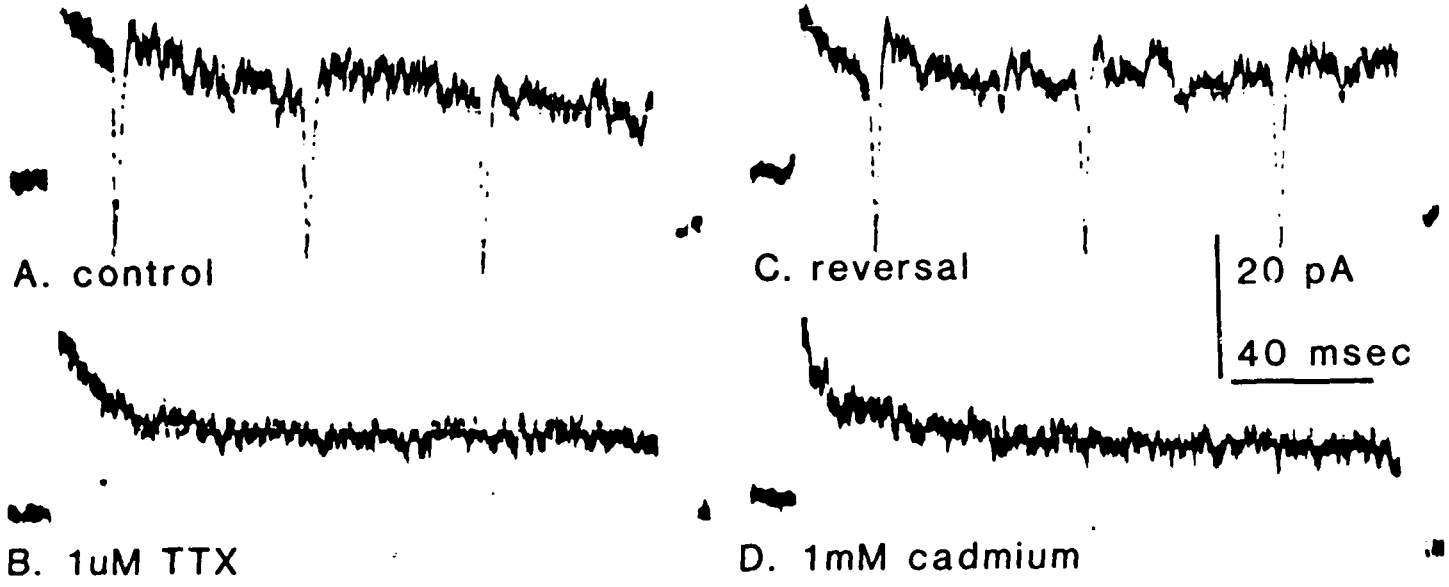
Fig. 2.

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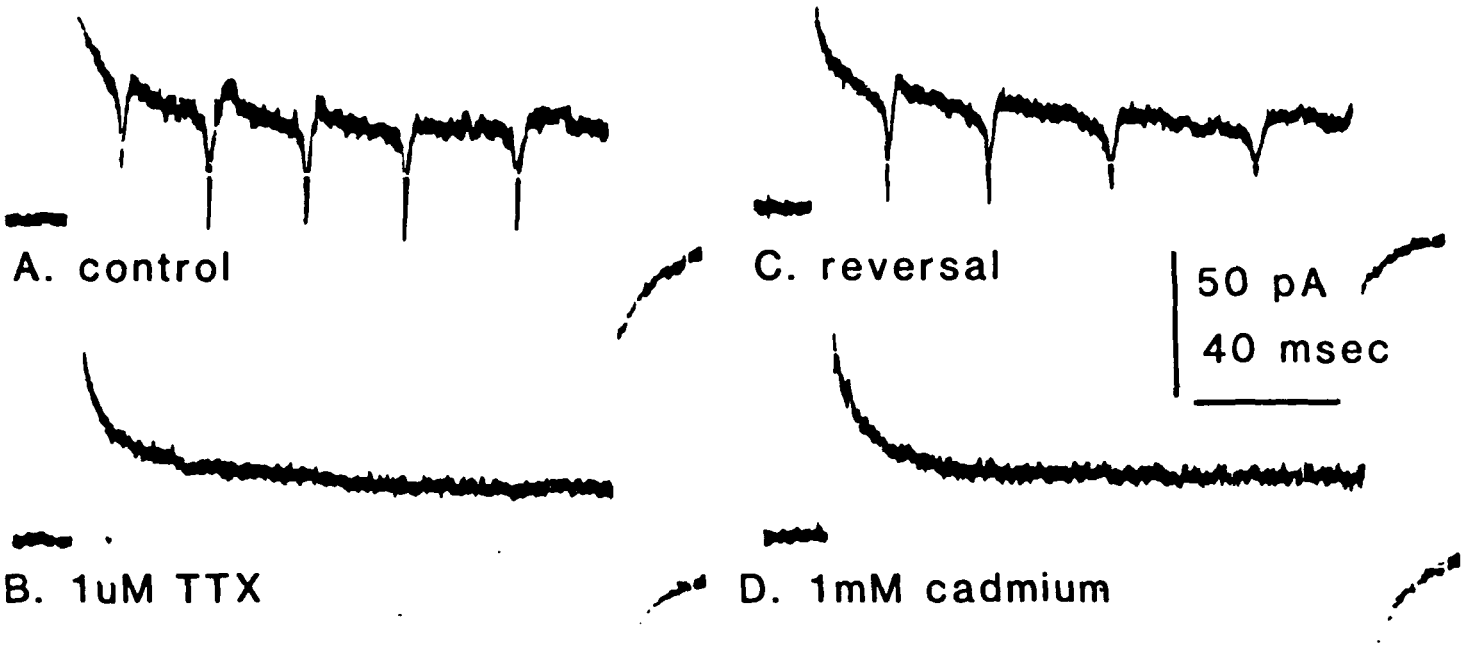


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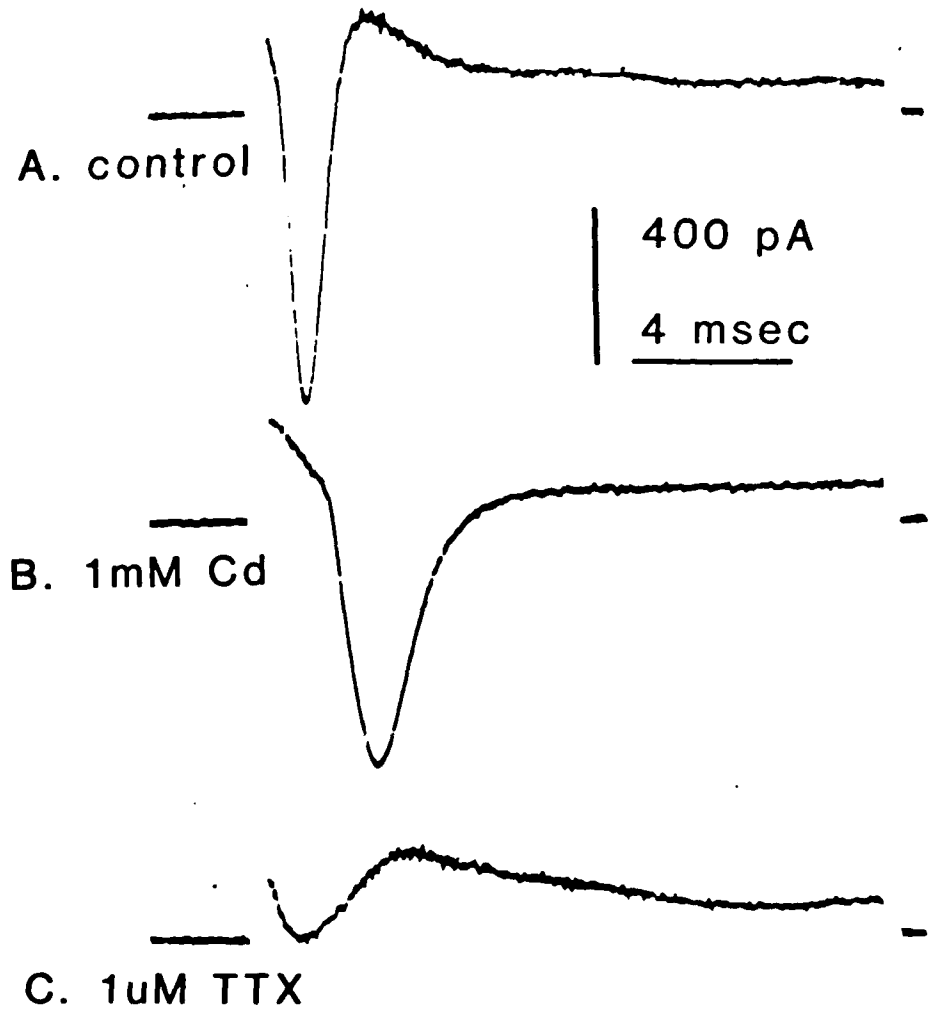


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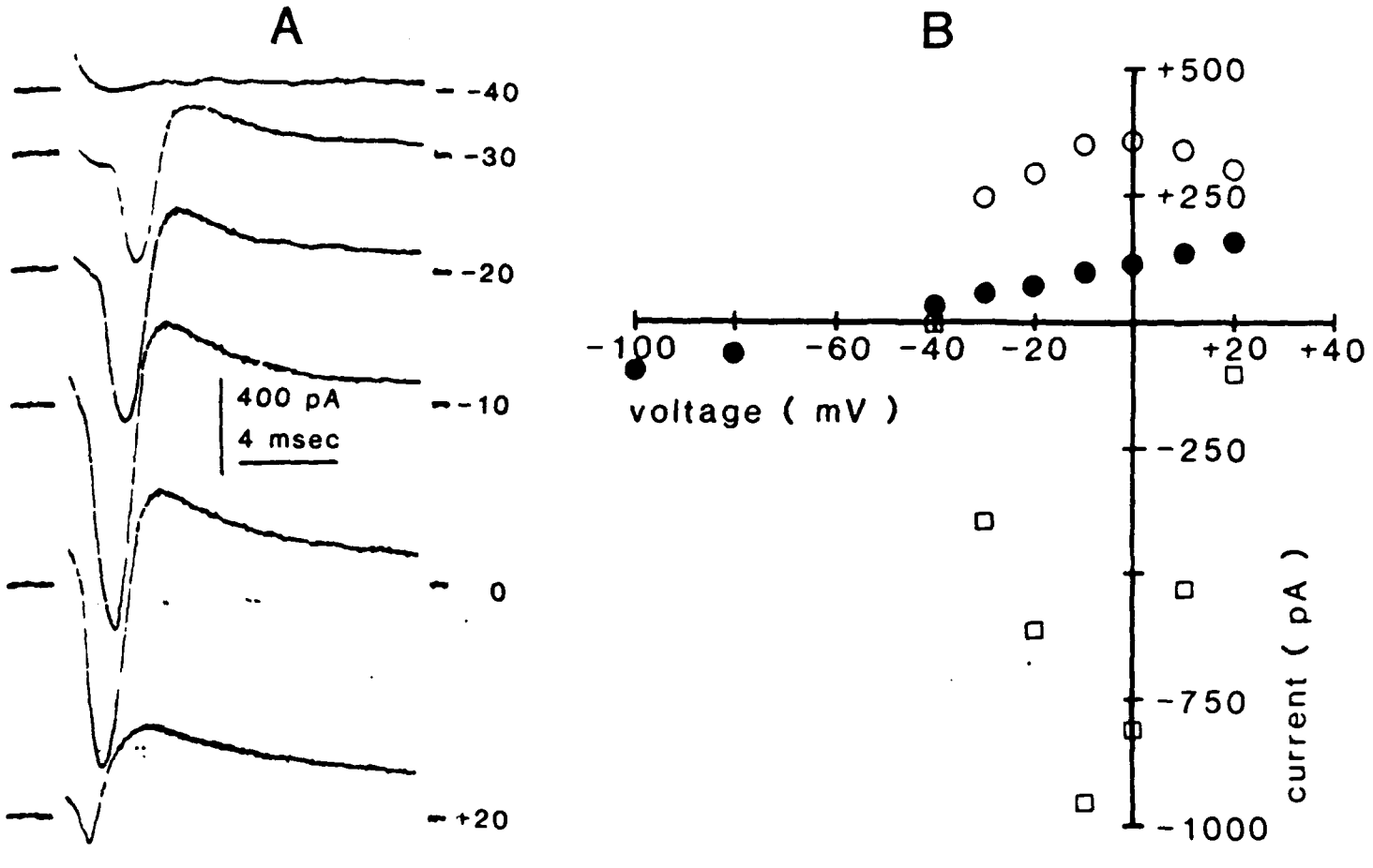
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Fig. 6.

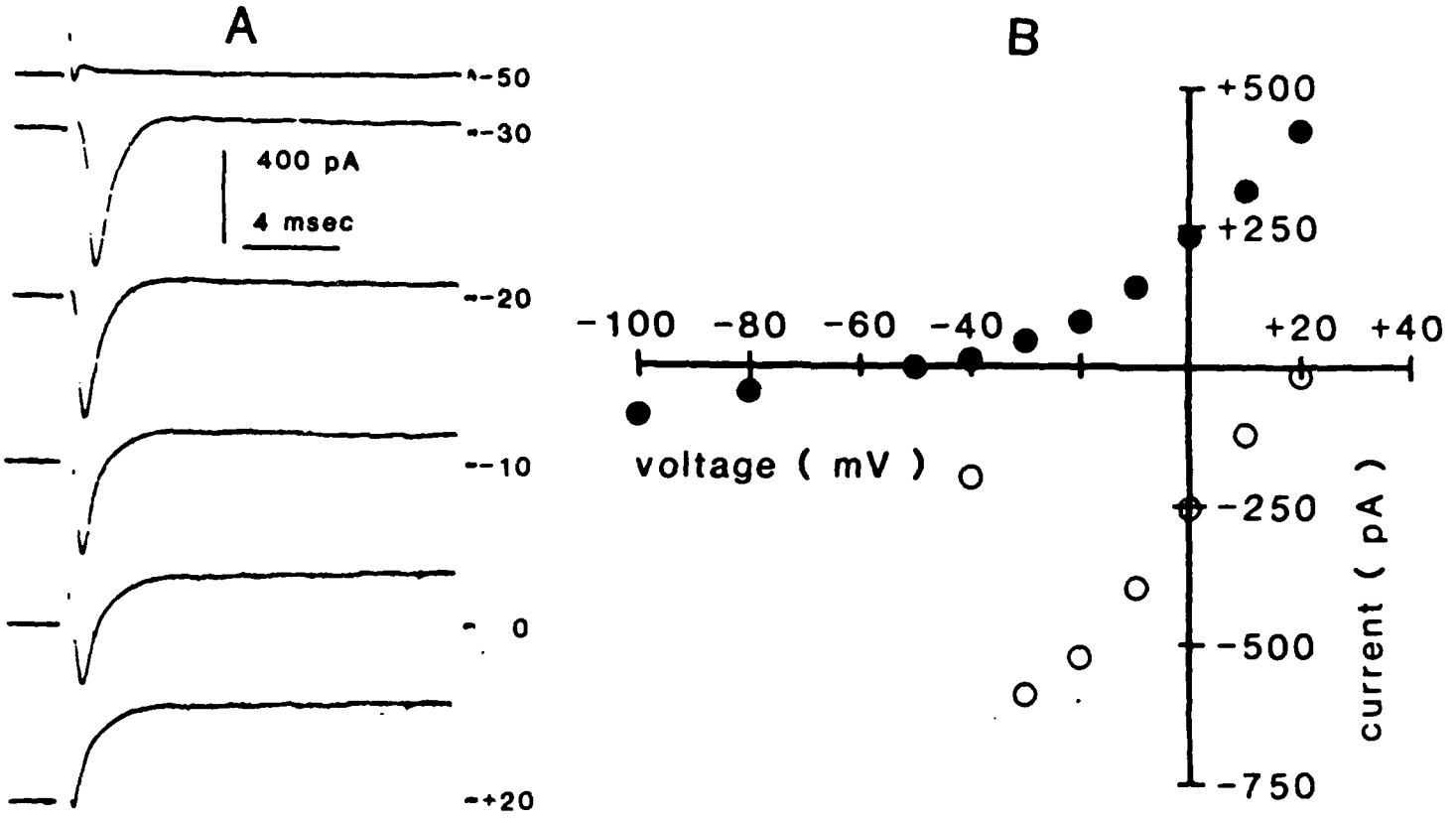
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Fig. 7.

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PROPERTIES OF CULTURED CEREBELLAR GRANULE CELLS MEASURED WITH WHOLE-CELL PATCH RECORDING AND IMAGING OF $Ca^{2+}$ INDICATOR FLUORESCENCE. P. E. Hockberger and J. A. Connor. Dept. Molecular Biophysics, AT&T Bell Laboratories, Murray Hill, NJ 07974.

In preparation for studies on the role of cyclic nucleotides in cerebellar granule cell function, we have begun to characterize the properties of these cells grown in vitro under completely defined conditions. Explants of P3 to P10 rat cerebellum were cultured at 37°C on polylysine-coated glass coverslips and fed 3X per week with DMEM plus defined additives as detailed elsewhere (Ahmed, Z. et al., J. Neurosci. 3:2448,1983). Granule-like cells were identified using several criteria including soma size and shape, dendritic morphology, birthdate, survival in $10^{-4}$ M kainic acid, and response to GABA iontophoresis (also see Messer, A., Brain Res. 130: 1,1977). Intracellular and voltage clamp data were obtained using the whole-cell patch recording technique.

Granule cells were identifiable after 2-3 days in culture and increased in number throughout the first week. During that time the cells exhibited progressive changes in several membrane properties. Membrane resistance dropped ($10^{-4}$ -> $10^{-3}$ $\Omega$ $cm^2$), resting potential increased (-30 -> -50 mV), and voltage-dependent conductances increased in amplitude. Small action potentials could be elicited after 7-10 days, and by 18 days in vitro overshooting action potentials were present. The following currents were recorded from these cells after 2-3 days in culture: (1) transient, 4-AP-sensitive outward current; (2) delayed outward current; (3) transient, TTX-sensitive inward current; and (4) GABA-activated current. The latter had a reversal potential around -50 mV (KAc-filled electrode) and was blocked by (+)-bicuculline or picrotoxin.

CCD imaging of cells loaded with the Ca-indicator dye fura-2 demonstrated that granule cells raised their cytoplasmic $Ca^{2+}$ levels when exposed to either high potassium (25 mM) or $10^{-5}$ M GABA. The GABA response persisted in the presence of 0.3 µM TTX, a concentration sufficient to eliminate the inward current of these cells. The source of the $Ca^{2+}$ elevation is presently under investigation.

That work not sponsored by AT&T Bell Laboratories was funded by a grant from AFOSR under contract F49620-85-C-0009.

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NEURONAL CaATPase ACTIVITY AND CHANGES IN INTRACELLULAR $Ca^{2+}$ INDUCED BY SHORT-TERM EXPOSURE TO THYROID HORMONE. L. Kragie-Ahmed*, Z. Ahmed, J. A. Connor and P. J. Davis*. Endocrin. Div. VAMC, Buffalo, NY 14215, Div. Neurobiol., SUNYAB, Buffalo, NY 14214 and Mol. Biophys., AT&T Bell Lab., Murray Hill, NJ 07974.

A thyroid hormone stimulable $Ca^{2+}$-dependent $Mg^{2+}$ ATPase activity (CaATPase) has been described in RBC, reticulocyte and sarcolemma preparations. Here, we report for the first time, stimulation of a CaATPase activity by thyroxin ($T_4$) and triiodothyronine ($T_3$) in a membrane preparation obtained from a primary neuronal culture grown in serum-free, defined medium. Using $Ca^{2+}$-sensitive fluorescent indicators, quin 2 and fura 2, we also found a decrease in intracellular calcium upon short-term exposure to $T_4$. Whole cell voltage clamp experiments showed that the presence of $10^{-9}$ M $T_3$ or $T_4$ in the bath did not alter the primary membrane currents.

Four to six day old cultured neurons from fetal rat diencephalon and cerebral cortex were subjected to hypotonic lysis and mechanical shear. After removal of the nuclei (700xg pellet) "membrane fraction" (10Kxg pellet) was resuspended and aliquoted into isotonic saline containing 0.1 mM oubain, 1 mg/ml oligomycin, 1 mM $MgCl_2$, 0.1 mM EGTA and ± 0.1 mM $CaCl_2$. The incubation was 60 min at 37°C. The concentration of hormone was varied between $10^{-11}$ to $10^{-8}$ M. Membrane CaATPase activity in the presence of $10^{-11}$ M $T_3$ was 140% greater than the no TH control. Peak activity in presence of $10^{-10}$ M $T_4$ was 60% greater than control. Similar data were also obtained from mature rabbit synaptic plasma membrane. The CaATPase activity seems to show differential sensitivity to $T_3$ and $T_4$ according to region of origin.

Cells growing on polylysine coverslips were loaded with either quin 2 or fura 2. Fluorescence of small groups of cells was imaged using a CCD-based system (see Connor, J.A., these abstracts). Following 5 to 10 min. exposures to $10^{-9}$ M $T_4$, $Ca^{2+}$ was significantly lower in a fraction of the cells on a given plate. In other cells on the same plate the $[Ca^{2+}]$ remained constant.

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# $Ca^{2+}$ MEASUREMENTS USING THE FLUORESCENT INDICATORS QUIN 2 AND FURA 2 COMBINED WITH DIGITAL IMAGING IN MAMMALLIAN CNS CELLS.


John A. Connor, Dept. Mol. Biophys., AT&T Bell Laboratories, Murray Hill, NJ 07974.


Cells in two types of culture were examined, trypsin dispersed cells from embryonic rat diencephalon (E17,18), and cerebellar explants (P3,8). In the explants, granule cells were the predominant type studied. Cells were loaded with indicator by bathing them (30 min., T=36°C) in defined media containing either 60 μM quin 2 or 8 μM fura 2. After loading and a 2 hr. post-load incubation, cells growing on #1 thickness coverslips were mounted on the stage of an inverted microscope and viewed through a 40X objective (1.3 N.A., glycerine). U.V. illumination was provided by a 100 w, short-arc Hg lamp. A charge coupled device (CCD) photometer was mounted at the 35 mm camera port of the microscope in the image focal plane. The CCD had 320 x 512 pixels, though generally, pixel binning was employed to reduce thermal noise variance (operating temperature -40 °C). Experimental procedure was to obtain sequential images (340 and 360 nm excitation for quin, 340 and 380 nm for fura) and then to form the ratio of the two images. The value of this ratio gives a measure of spatially resolved $Ca^{2+}$ concentration independent of indicator concentration and cell geometry to a first approximation. Exposure times of .15 to .25 s were employed for each image and a temporal separation of about 1 sec was required to change the excitation filter. In dispersed cultures 5 to 15 cells could be included in a given field, while for small, densely packed granule cells from explants, the number was often greater than 20.

In the dispersed cultures there were large differences in the ratio images of the various cells in a given field (>100%), while there was uniformity to within a few percent in granule cell populations. Exposure to high K saline (25 mM) produced measurable increases in $Ca^{2+}$ within 30 sec to 1 min. Only a fraction of the cells in diencephalon cultures responded to high K. Addition of TTX (.3 μM) to high K saline caused a rapid decrease in $Ca^{2+}$ indicating that the increase was due in some cases to enhanced action potential firing. Nitrendipine and nifedipine (10 μM) caused a decrease in $Ca^{2+}$ in subpopulations of the diencephalon cells, but the cell types affected have not been worked out at this time. Cells new in culture (growing) showed higher levels of $Ca^{2+}$ than cells of more mature cultures. Examples of growth cones with extended filapodia have been observed. In these cases, $Ca^{2+}$ in the cone was generally higher than in the soma. Where filapodia were not evident the ratio image was nearly uniform throughout the cell.

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## MEASUREMENT OF CYCLIC AMP CONCENTRATION IN *APLYSIA* NERVE CELL BODIES.

P. Hockberger and T. Yamane.* *Dept. Molecular Biophysics, AT&T Bell Laboratories, Murray Hill, NJ 07974.*

Several investigators have measured basal as well as stimulated levels of cyclic AMP in giant *Aplysia* neurons (Cedar & Schwartz, *J. Gen. Physiol., 60:* 570, 1972; Levitan & Norman, *Brain Res., 187:* 415, 1980; Bernier, Castellucci, Kandel & Schwartz, *J. Neurosci., 2:* 1682, 1982). However in each case the reported values were expressed as total cyclic AMP content per cell. Since the cell bodies are not uniform in size or shape, we have measured cyclic AMP levels normalized to both cell body volume and protein content. Using the single cell isolation procedures of Bernier et al., we have analyzed *Aplysia* cells $R_2$, LP1, and $L_2$-$L_6$ cluster, as well as whole ganglia. Cell body volumes were estimated assuming oblate spheroid shapes and using an average cell diameter computed by measuring the major and minor axes of each cell body. Protein content was determined using a fluorescamine method (Roche Diagnostics) as well as Lowry determinations. Cyclic AMP levels were measured using either a protein-binding radioassay (Amersham) or radioimmunoassay (New England Nuclear).

Table 1. Mean values (±SEM) of cyclic AMP concentrations in isolated *Aplysia* cells and desheathed abdominal ganglia (minus bag cells and connectives).

| COMPONENT | μM | p moles/mg. protein |
|---|---|---|
| Cell $R_2$ (n=9) | 16.3 ± 3.8 | 460 ± 87 |
| LP1 (n=10) | 17.9 ± 3.0 | 424 ± 126 |
| $L_2$ - $L_6$ (n=8) | 25.8 ± 4.8 | 468 ± 111 |
| Abd. ganglia (n=4) | XX | 16.4 ± 5.7 (S.D.) |

Our preliminary results are shown in Table 1. We have not attempted to distinguish between nuclear and cytoplasmic content of cyclic AMP. Also, the glial coat which surrounds even isolated cells contributes additional uncertainty to our measurements. Nevertheless, our values of total cyclic AMP content/mg. protein for *Aplysia* abdominal ganglia are similar to those reported by Treistman & Levitan (*Nature 261:* 62, 1976). Our range of values for total cyclic AMP content per cell (not shown) is also similar to those found by both Cedar & Schwartz (1972) and Levitan & Norman (1980). In fact if one estimates the cellular diameters to have been 400-500 μm in the cells $R_2$ and LP1 that they examined, then basal cyclic AMP concentrations were 5-10 μM per cell in those studies similar to the values we report here.

The cellular concentration of cyclic AMP in *Aplysia* neurons appears to be similar to cyclic GMP levels in isolated rod outer segments (Woodruff, Bownds, Green, Morrisey & Shedlovsky, *J. Gen. Physiol. 69:* 667, 1977) where levels reach 40-80 μM in the dark-adapted retina. In both *Aplysia* neurons (Connor & Hockberger, *J. Physiol.* in press) and photoreceptors (Miller & Nicol, *Nature 280:* 64, 1979) fluctuations in the resting level of as little as 30 μM cyclic nucleotide results in membrane depolarization of several millivolts. The results presented here suggest that, as in photoreceptors, physiological control of membrane potential in *Aplysia* cells may be regulated by enzymes with $K_m$'s for cyclic nucleotides in the μM range.

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